

Kinetics of serological responses in influenza A(H7N9)-infected patients correlate with clinical outcome in China, 2013

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The novel avian influenza A(H7N9) infection has recently emerged to cause severe respiratory illness in China. The objectives of this study were to define the kinetics of the antibody responses in patients with influenza A(H7N9) disease and to correlate these kinetics with clinical outcome. Serial serum samples were obtained at intervals of three to four days from 18 patients with virologically confirmed A(H7N9) disease in Shanghai. We determined the kinetics of the haemagglutination inhibition (HI) and A(H7N9) pseudotype neutralisation antibody (Nab) responses and correlated these with clinical outcomes. Most patients had robust serological responses by both HI and Nab tests. Taking into account censoring due to time of testing and death, the median time from onset of illness to Nab titre $\geq 1:40$ was 14 days (95% confidence interval (CI): 11–18 days) in the fatal cases and 10.5 days (95% CI: 7–12) in the survivors ($p=0.003$). The two groups did not differ in initial Nab titres, but the rate of increase in Nab titres was significantly faster for survivors by approximately 10-fold per 15 days ($p=0.007$). Early and rapid induction of Nab was correlated significantly with better clinical outcome.

Introduction

A novel avian influenza A(H7N9) infection emerged to cause an outbreak in the Yangtze River delta in early 2013, subsequently spreading to other provinces in China [1–4]. In the first wave of influenza A(H7N9) infections from February to July 2013, 135 patients were reported from 11 provinces and municipalities in China, leading to 45 deaths [5]. Further cases have been reported since October 2013. Genomic analysis revealed that the novel H7 haemagglutinin is genetically distinct from other historical and contemporary human influenza viruses [3,6]. Adverse clinical outcomes have been associated with co-existing medical conditions or the development of drug resistance [7,8]. Previous

H7 subtype influenza virus infections in humans such as the influenza A(H7N7) outbreak in Netherlands in 2003 were poorly immunogenic and serodiagnosis and seroepidemiology were challenging [9]. We explored the kinetics of the serological responses to this novel virus in haemagglutination inhibition (HI) assays and in a recently developed H7N9 pseudotype virus particle neutralisation (Nab) test [10]. Viral pseudotypes have been previously shown to provide reliable correlation with conventional microneutralisation tests for influenza A(H5N1) serological studies [11]. We investigated correlations between serological responses and clinical outcome.

Methods

Patients and samples

In April 2013, 18 patients confirmed with influenza A(H7N9) infection by real-time PCR were hospitalised at the Shanghai Public Health Clinical Center (SHAPHC). Serum specimens were collected every three to four days following admission with two to seven serial serum samples being collected from each patient. Clinical data including patient demographic information, treatment, clinical investigations and disease progression were retrieved from the clinical notes. Written informed consent was obtained from all participants. The overall study was reviewed and approved by the Ethics Committee of SHAPHC.

Haemagglutination inhibition assay

The methods used were as previously described and used horse erythrocytes [12]. Serum samples were treated with receptor-destroying enzyme (RDE) (Denka Seiken Co Ltd., Tokyo, Japan) to remove non-specific inhibitors. Stored serum samples collected in 2009 from individuals not infected with influenza were used as negative serum controls. The virus strain used was

A/Shanghai/4664T/2013 (H7N9) (GenBank accession No: KC853228.1).

Pseudovirus-based neutralisation assay

To rapidly and safely assess neutralisation activities against the 2013 influenza A(H7N9) virus which caused severe disease in humans, we developed a luciferase reporter-based Nab assay which has a non-replicative human immunodeficiency virus backbone carrying influenza A H7 and H9. We have previously demonstrated that the titres quantified by Nab assay correlated well with the titres measured by traditional HI assay, using serum samples from influenza A(H7N9)-infected patients and uninfected subjects with good correlation (Spearman $r=0.88$) [10]. The pseudoviruses were prepared as described in our previous report [10]. The neutralising titre of human sera was defined as the highest serum dilution that gave $\geq 80\%$ inhibitory concentration (IC₈₀) of the luciferase signal in virus-infected MDCK cells. On the basis of previous studies we had defined that IC₈₀ and a antibody titre of 1:40 were the best discriminators between patients and non-infected controls, and we employed these to define positive Nab responses.

Statistical analyses

Non-parametric Mann–Whitney test was used to test the differences in HI or Nab titres across groups. Categorical variables were compared by using the two-tailed Fisher's exact test to account for small sample size. In addition, univariate and multivariate exact logistic regression modelling were employed to identify the association of different factors with clinical outcome and allow for small cell size. The covariates used in the multivariate model included age, sex and Nab titres (1:40 and 1:640). The results were presented using odds ratios (ORs).

We fitted accelerated failure time model assuming a Gaussian distribution to compare time from illness onset to reaching a Nab titre of 1:40 between patients who recovered versus those who died, accounting for interval censoring due to time of testing. The model was also used to identify factors associated with longer time to recovery for recovered patients. We compared the initial Nab titre and rate of increase in Nab titre, adjusted for age and sex, using a linear mixed model to account for repeated measurements, assuming a linear increasing trend by days since illness onset. For analyses based on continuous measurements, titres were first log-transformed (with base 10). We used bootstrap method with 1,000 resamples to test the difference in time from illness onset to reaching a Nab titre of 1:40 between fatal cases and survivors. All statistical tests were considered significant at the level of $p<0.05$. All data were analysed by using SPSS software (version 17.0) and R (version 3.0.1).

Results

To understand the kinetics of the human serological responses to the novel influenza A(H7N9) virus, we

first determined (HI) antibody and pseudotype Nab responses in 18 influenza A(H7N9)-infected patients. HI antibody titres reached a titre of 1:40 in six of 14 patients by Day 10 of illness. By Day 18 of illness, 17 of 18 patients had antibody titres of 1:40 with titres ranging from $<1:10$ to 1:320, and 10 of 18 patients had titres $\geq 1:80$. All patients had evidence of seroconversion within three weeks (Figure). We next examined Nab titres quantified by the Nab assay. Nab titres reached 1:40 in five of 14 patients by Day 10 of illness and 16 of 17 by Day 18 of illness. Thirty-seven control sera collected in 2009 had negative results in both tests. To test the reliability of assays, limited repeat testing has been done on sets of sera by both assays with good reproducibility; in addition, all the sera were tested in one large assay batch to maintain comparability.

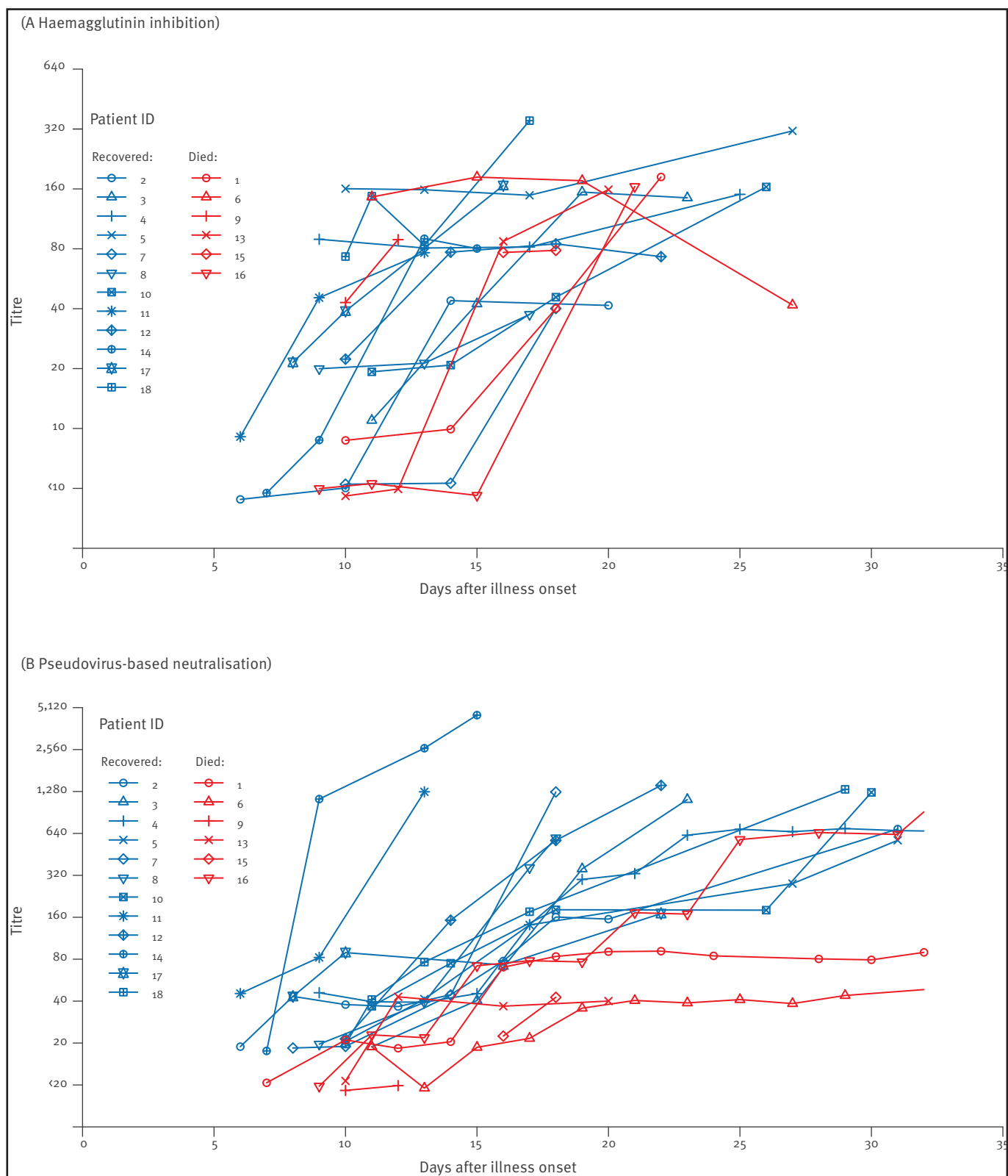
We examined the association between HI and Nab responses with clinical outcomes. The 18 patients were divided into two groups: group A were 12 patients who recovered and group B were six patients who had died (Table 1). The differences in HI titres between the two groups were not statistically significant. We next examined the development of Nab in the two patient groups, taking into account censoring due to time of testing and death. The median time from symptom onset to Nab titres reaching 1:40 was 10.5 days (95% confidence interval (CI): 7–12 days) for patients who survived and 14.0 days (95% CI: 11–18 days) for those who died. In patients who survived, it took a significantly shorter time to reach Nab titres of 1:40 for the unadjusted model ($p<0.001$) and the model adjusted for age and sex ($p<0.001$). Adjusted for age and sex, there was no significant difference ($p=0.36$) in the initial Nab titre between patients who survived and those who died. However, the Nab titre for survived patients increased significantly faster than for patients who eventually died, by approximately 10-fold per 15 days ($p=0.007$). The difference persisted when we restricted the analysis to patients with low initial Nab titres ($\leq 1:40$ on day 10, $p=0.003$).

We further examined the association between clinical outcome and sex, age, underlying medical disorders, timing for initiating antiviral treatment with oseltamivir or anti-inflammatory treatment with methylprednisolone and initial viral loads. None of these factors were significantly associated with clinical outcomes in the univariate analysis (Table 2). Nab titres reaching $\geq 1:640$ or reaching 1:40 within 14 days were associated with survival in both univariate and multivariate analysis (Table 2). However, HI titres reaching 1:40 within 10 days were not associated with survival.

We also examined the association of these different factors with time to recovery in the survivors. Reaching Nab titres 1:640 within 14 days after illness onset was significantly associated with faster recovery ($p=0.002$) in the univariate analysis (Table 3). After adjusting for other confounding factors, it was associated with a 22% shorter recovery time, although the effect was not

FIGURE

Antibody responses in influenza A (H7N9) patients after illness onset, China, 2013 (n=18)



Serum samples were collected from all 18 influenza A(H7N9)-infected patients. For each patient, two to seven serial serum samples at intervals of three to four days were collected after their admission to our center. The population level kinetics of both (A) HI and (B) neutralisation antibody responses of each individual patient are shown over time. HI titres were not significantly different between survivors and fatal cases at eight to 10 days ($p=0.126$) and at 11 to 14 days ($p=0.390$) after illness onset.

TABLE 1

Viral loads and clinical outcomes in influenza A (H7N9)-infected patients, China, 2013 (n=18)

	Patient number	Sex	Age	Underlying medical disorders	Time between onset of symptoms and		Initial viral loads (copies/mL)	Clinical outcome
					the initiation of oseltamivir treatment (days)	first treatment with methylprednisolone (days)		Discharged after days of onset or death
Group A: recovered	11	M	53	None	5	5	6.59x10 ²	14
	14	M	47	None	5	5	9.30x10 ³	17
	7	M	68	Hypertension II	6	No	1.29x10 ³	18
	8	M	65	Hypertension, urethritis	4	No	3.49x10 ²	18
	17	F	74	Hypertension III, coronary heart disease (NYHA IV), diabetes II, cholecystitis	8	No	5.11x10 ³	21
	12	M	74	None	8	12	3.68x10 ²	22
	2	F	81	Arrhythmia	5	6	3.08x10 ³	23
	3	M	67	None	5	4	4.24x10 ³	23
	18	M	67	Diabetes II	11	9	3.86x10 ²	24
	10	M	78	Hypertension II	11	15	2.28x10 ²	31
	5	F	75	Hypertension II, coronary heart disease (NYHA IV), diabetes	8	9	1.13x10 ³	33
	4	M	62	Hypertension	9	5	5.70x10 ⁵	35
Group B ^a : died	16	F	79	Arrhythmia, coronary heart disease (NYHA IV), chronic bronchitis, primary biliary cirrhosis	6	6	5.65x10 ⁴	Death
	1	M	56	None	3	7	2.67x10 ²	Death
	6	M	58	Hypertension	9	8	Negative	Death
	13	M	88	Hypertension III, coronary heart disease (NYHA IV), diabetes II, chronic bronchitis	7	8	4.06x10 ⁴	Death
	15	M	80	None	7	7	5.8x10 ³	Death
	9	M	74	Coronary heart disease (NYHA III)	6	6	Negative	Death

F: female; M: male; NYHA: New York Heart Association Functional Classification.

^a The virus isolates in patients 13 and 1 developed the Arg292Lys mutation in the neuraminidase protein, which is known to confer resistance to oseltamivir.

Patients are listed by date of discharge from our hospital.

statistically significant ($p=0.108$). Only age and time until oseltamivir treatment remained significant in the multivariate analysis (Table 3).

To specifically examine whether use of methyl prednisolone had an impact on antibody responses, we fitted a linear mixed model including 'initiation of methylprednisolone treatment less than eight days after disease onset' as a variable to predict trends in HI and Nab titres. Adjusted for age and sex, receiving methylprednisolone treatment within eight days did not have a significant influence on HI and Nab titres.

Discussion

The kinetics of the antibody responses in human A(H7N9) virus infections remain ill defined. Such data are important for serodiagnostic purposes as well as for the interpretation of seroepidemiological studies. Our findings indicate that influenza A(H7N9) infections are associated with generally robust HI antibody responses and, with one exception (Patient 9, a patient who died), Nab antibody responses. Therefore, serodiagnosis of hospitalised patients should not pose a major challenge, unlike human H7N7 infections in the Netherlands in 2003 [9]. It is relevant to note that

TABLE 2

Association of different factors with clinical outcomes in influenza A (H7N9)-infected patients, China, 2013 (n=18)

Variables	Discharge n (%)	Death n (%)	Univariate analysis		Multivariate analysis	
			OR ^a (95% CI)	p value	OR ^a (95% CI)	p value
Sex						
Male	9 (64.3)	5 (35.7)	1	–	–	–
Female	3 (75.0)	1 (25.0)	1.62 (0.096–105)	1	–	–
Age						
<75 years	9 (75.0)	3 (25.0)	1	–	–	–
≥75 years	3 (50.0)	3 (50.0)	0.344 (0.027–4.17)	0.356	–	–
Medical disorders						
No	4 (44.7)	2 (33.3)	1	–	–	–
Yes	8 (66.7)	4 (33.3)	1.00 (0.064–11.5)	1	–	–
Initiation of oseltamivir treatment						
<8 days	6 (54.5)	5 (45.5)	1	–	–	–
≥8 days	6 (85.7)	1 (14.3)	4.59 (0.346–275)	0.316	–	–
Initiation of methylprednisolone treatment						
<8 days	5 (55.6)	4 (44.4)	1	–	–	–
≥8 days	7 (77.8)	2 (22.2)	2.64 (0.253–40.5)	0.620	–	–
First viral load						
<1,000 copies/mL	5 (62.5)	3 (37.5)	1	–	–	–
≥1,000 copies/mL	7 (70.0)	3 (30.0)	1.37 (0.126–15.2)	1	–	–
Nab titre reaches 1:640						
No	1 (16.7)	5 (83.3)	1	–	1	–
Yes	11 (91.7)	1 (8.3)	35.4 (1.99–2740)	0.004	16.9 (2.19–220)	0.006
Nab titre reaches 1:40						
≤14 days	11 (84.6)	2 (15.4)	1	–	1	–
>14 days	1 (20.0)	4 (80.0)	0.0587 (0.000858–0.941)	0.022	0.0361 (0.000–0.519)	0.011
HI titre reaches 1:40 ^b						
≤10 days	5 (62.5)	3 (37.5)	1	–	–	–
>10 days	7 (70.0)	3 (30.0)	1.37 (0.126–15.2)	1	–	–

–, not relevant; CI: confidence interval; HI: haemagglutination inhibition; Nab: pseudotype neutralisation antibody; OR: odds ratio.

^a OR>1 indicates 'preferring recovery'. Multivariate analyses were performed by examining whether Nab titres reached 1:640 or 1:40 within 14 days in a model with co-factors of age and sex.^b Missing HI titres within 10 days were imputed conservatively in such a way that would attenuate the results toward the null hypothesis.

nearly all diagnosed H7N9-infected patients had more severe disease (namely pneumonia) compared with the outbreak in the Netherlands, where diagnosed patients had mainly mild conjunctivitis or influenza-like illness. Thus, one cannot be certain that milder or asymptomatic influenza A(H7N9) infections would manifest comparably robust serological responses. In addition, this has to be kept in mind when interpreting community-wide seroepidemiological studies. It would be interesting to have serological data from family members and contacts of our patients. Unfortunately, such a follow-up study was not conducted.

The progression of influenza A(H7N9) disease was observed to be slower than of influenza A(H5N1) disease, the median time from hospitalisation to death being 12.0 days and 5.7 days, respectively [13]. We hypothesised that the prolonged disease course of H7N9 compared with H5N1 influenza (and SARS-CoV) infection is likely to imply that there is more opportunity for the adaptive immune response to contribute to recovery and survival. Therefore, we examined the antibody responses with HI and Nab assays in influenza A(H7N9)-infected patients admitted into our clinical center. We evaluated the correlation between the kinetics of HI and Nab antibody responses and clinical

TABLE 3

The association of different factors with time to recovery for recovered patients

Variables	Median time to recovery (days)	Univariate analysis		Multivariate analysis	
		AF ^a (95% CI)	p value	AF ^b (95% CI)	p value
Sex					
Male	22.0	1		1	
Female	23.0	1.17 (0.831–1.64)	0.374	0.893 (0.709–1.12)	0.332
Age					
<75 years	21.0	1		1	
≥75 years	31.0	1.39 (1.03–1.86)	0.031	1.32 (1.05–1.66)	0.019
Medical disorders					
No	19.5	1		1	
Yes	23.5	1.32 (0.999–1.75)	0.051	0.994 (0.795–1.24)	0.960
Initiation of oseltamivir treatment					
<8 days	18.0	1		1	
≥8 days	27.5	1.46 (1.18–1.81)	<0.001	1.31 (1.11–1.56)	0.002
Initiation of methylprednisolone treatment					
<8 days	23.0	1		–	–
≥8 days	22.0	1.09 (0.806–1.48)	0.575	–	–
First viral load					
<1 000	22.0	1		–	–
≥1 000	23.0	1.12 (0.826–1.51)	0.476	–	–
Nab titre reaches 1:640					
>21 days or not reaching 1:640	23.5	1		–	–
15–21 days	20.0	0.878 (0.681–1.13)	0.312	0.996 (0.803–1.23)	0.968
≤14 days	15.5	0.606 (0.440–0.835)	0.002	0.784 (0.584–1.05)	0.108
Nab titre reaches 1:40					
≤14 days	22.0	1		–	–
>14 days	23.0	1.03 (0.595–1.78)	0.922	–	–
HI titre reaches 1:40 ^b					
≤10 days	24.0	1		–	–
>10 days	22.0	0.88 (0.655–1.191)	0.415	–	–

–, not relevant; CI: confidence interval; HI: haemagglutination inhibition; Nab: pseudotype neutralisation antibody; AF: acceleration factor.

^a AF<1 indicates reduction in time from illness onset to recovery. Multivariate analyses were performed by examining medical disorder, initiation of oseltamivir treatment after eight days and time to reach Nab titres 1:640, in a model with co-factors of age and sex.^b Missing HI titres within 10 days were imputed conservatively in such a way that would attenuate the results toward the null hypothesis.

disease outcome of survival or death. Strikingly, we observed that the early and rapid induction of Nab significantly correlated with the rapid recovery from illness. The two groups of patients did not differ in initial Nab antibody titres, but the rate of antibody titre increase was approximately 10-fold faster per 15 days in survivors than in fatal cases ($p=0.007$). In contrast, no significant association was observed between HI titres and clinical outcomes. While the HI test only detects antibodies against the receptor-binding domain of the globular head of the haemagglutinin, neutralisation could be conferred by antibodies against the stalk

of the haemagglutinin as well as the neuraminidase. Therefore, it is possible that neutralisation and HI antibodies may differ in their capacity to control influenza infection, and Nab may represent a better early surrogate marker for recovery from H7N9 influenza disease.

In those who survived the infection, reaching Nab titres of ≥1:640 within 14 days of illness was associated with a trend toward faster recovery. Interestingly, while age and time to oseltamivir treatment were not significantly associated with survival in the group overall, they were associated with faster recovery in the survivors. This

difference may be related to the finding that emergence of oseltamivir resistance was associated with adverse clinical outcome in at least two of our patients with fatal outcome [8]. Another recent study reported association between faster serological responses and survival in H7N9 influenza patients, although it did not account for censoring due to time of testing and death, nor investigate the role of other confounding factors such as corticosteroid therapy or antiviral therapy [17].

Antibodies use different mechanisms to contain virus infection, including direct neutralisation of virus entry, antibody-dependent cytotoxic responses and directing presentation of virus–antibody complexes to antigen-presenting cells [14]. It is also possible that the early development of Nab antibodies and the rapid involvement of Nab antibodies in responding to influenza virus infection may alter the innate immune response and thereby result in a milder disease course. Previous reports have demonstrated that severe clinical disease in highly pathogenic avian influenza infection was usually associated with skewed innate immune responses which were characterised by the production of more pro-inflammatory cytokines and less type I interferon, whereas a mild influenza disease course was associated with a balanced type I interferon and inflammatory response [15,16]. An alternative explanation is that the antibody responses are a surrogate marker for cell-mediated immune responses.

In summary, our data demonstrate that the haemagglutinin of the 2013 A(H7N9) influenza virus is immunogenic and capable of rapidly eliciting robust HI and Nab antibody responses. The kinetics of the Nab response are correlated with clinical outcome. Cause and effect cannot yet be established because antibody responses may be a confounding factor associated with other protective determinants of clinical outcome. Convalescent plasma therapy has previously been shown to reduce mortality in patients in intensive care with pandemic H1N1 influenza [18]. Thus, our data may indicate that early passive antibody therapy may be beneficial to clinical outcome, especially in those who fail to mount rapid Nab antibody responses.

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Conflict of interest

None declared.

Authors' contributions

AZ, YH, DT, EL, YW and XL conducted the study and analysed data, ZS, YD, XZ, JZ, MB, MZ, SY, JS, ZZ, YWH, LC, CL and JW processed samples and prepared sera samples., XYZ and ZYZ coordinated the study and provided samples, MP and JX conceived and designed the study, analysed data and wrote the manuscript.

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